Final Scientific Report

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BARD Project Number: IS-4234-09

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Project Title: Understanding Cuticle Development in Tomato through the Study of Novel Germplasm with Malformed Cuticles

Investigators	**Institutions**
Principal Investigator (PI): Arthur A. Schaffer	**ARO, Min. Ag.**
Co-Principal Investigator (Co-PI): Jocelyn Rose	**Cornell U.**

Keywords *not* appearing in the title and in order of importance. Cuticle mutants, transcriptome, proteome

Abbreviations commonly used in the report, in alphabetical order: cd, cuticle deficient; cwp, cuticular water permeability

Budget: IS: $ 165,000 US: $ 165,000 Total: $ 330,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	1	3		4
Submitted, in review, in preparation	1	1	2	4
Invited review papers		1		1
Book chapters		1		1
Books				
Master theses				
Ph.D. theses			1	1
Abstracts			3	3
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training:

List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	1		2
Longer Visits (Sabbaticals)				

Description Cooperation:

During the research project we cooperated in the development of biological material, especially for the scanning electron microscope (SEM) study of cuticles from the wild species, from which a joint publication was prepared. In addition, transcriptomic data generated in the Rose lab, and proteomic data generated in the Schaffer lab were interchanged, leading to the characterization of the fruit cuticle proteome and the cellular origin of the related transcripts and this joint publication is in preparation.

Furthermore, frequent communication by e-mail was carried out in order to update each other on research progress and reciprocal visits of the PIs were especially fruitful.

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted		1		1
Issued (allowed)				
Licensed				

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Understanding Cuticle Development in Tomato through the Study of Novel Germplasm with Malformed Cuticles

Abstract

Plant cuticle development and metabolism are still poorly understood, partly due to the chemical complexity of the cuticular layer. The overall research objective was to broaden and deepen our understanding of tomato fruit cuticle development by analyzing novel germplasm with cuticular malformations and by studying the transcriptome and proteome of the fruit epidermal tissues, as strategies to overcome the challenges posed by the recalcitrance of the biological system.

During the project we succeeded in identifying two genes with major impact on cuticle development. One of these encoded the first cutin synthase to be identified in plants, a metabolic step that had been a black box in cutin synthesis. In addition genes controlling the triterpenoid components of the cuticle were identified and, most interestingly, genetic variability for this component was identified among the wild tomato species germplasm. Additional germplasm was developed based on interspecific crosses that will allow for the future characterization of modifier genes that interact with the microfissuring gene (*CWP*) to promote or inhibit fruit cracking. One of the major accomplishments of the joint project was the integrated transcriptomic and proteomic analysis of the fruit cuticle and underlying tissues which allows for the identification of the pericarp cell layers responsible for the extracellular, cuticle-localized protein component.

The results of the project have expanded our understanding of tomato fruit cuticle development and its genetic control. In addition, germplasm developed will be useful in developing tomato varieties resistant to cracking, on the one hand, and varieties useful for the dehydration industry on the other.

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Evaluation of the research achievements as relates to the original research proposal and objectives.

We successfully cloned and characterized two genes whose mutant forms lead to simply inherited cuticle malformation phenotypes. The *cm17* gene, which we renamed *hyper-cracking 1* (*HCR1*), was predicted to encode a bifunctional enzyme; 3β-hydroxysteroid dehydrogenase/C-4 decarboxylase (3β-HSD/D1) of the steroid biosynthetic pathway. Sterol profiling showed that the mutation results in a significant reduction in sterol levels in *hcr1* mutant fruits. Characterization of the *hcr1* mutant using morphological, cytological and molecular approaches showed that the loss-of-function of the 3β-HSD/D1 enzyme results in an inhibition of cell expansion and division in the *hcr1* fruit pericarp. Our data suggest that the massive fruit cracking results from an uncoordinated tissue growth rate between the pericarp and the inner tissue due to the limited growth potential of the pericarp. Analysis of major polysaccharide components of the pericarp cell wall showed that *hcr1* mutant exhibits a major cellulose deficiency, while pectin levels were normal. This result underscores the importance of sterols in cellulose biosynthesis.

In addition, we mapped the *CD1* gene and showed that the locus is predicted to encode a GDSL-motif lipase/hydrolase family protein, a superfamily of proteins that includes both predominant lipases and acyltransferases. In the wild type fruit, cutin is abundant and composed primarily of inter-esterified monomers of 10,16-dihydroxyhexadecanoic acid (DHHA). However, fruit of the *cd1* tomato mutant show a severe reduction (>95%) in the amount of polymerized cutin, but accumulate the soluble glyceryl ester of the cutin monomer, 2-mono-(10,16-dihydroxyhexadecanoyl)glycerol (2-MHG). A long-standing question in plant lipid biology has been the mechanism and location of cutin polymerization, particularly given its insoluble and extracellular nature. In vitro, CD1 catalyzes the formation of ester oligomers from 2-MHG, suggesting that cutin polymerization *in planta* proceeds via successive rounds of transesterification, leading to esterified DHHA groups and the release of free glycerol. Thus we concluded that CD1 is a cutin synthase that catalyzes the formation of 95% of the tomato fruit cutin.

We performed additional biochemical characterization of CD1 and putative orthologs from *Arabidopsis thaliana* and the moss *Physcomitrella patens*. We demonstrated that members of this ancient and conserved family of cutin synthase (CUS) proteins act as polyester synthases. Moreover, solution-state NMR analysis indicates that CD1 catalyzes the formation of primarily linear cutin oligomeric products. These results reveal a conserved mechanism of cutin polyester synthesis in land plants.

Remarkable species diversity in the structure and composition of the cuticle components, cutin and wax, have been catalogued, but few functional or genetic correlations have emerged. With the aim of evaluating the relationships between evolution, structure and function of the cuticle, we characterized the morphological and chemical diversity of fruit cuticles of seven species from *Solanum* Sect. *Lycopersicon*. Striking differences in cuticular architecture and quantities of cutin and waxes were observed, with wild species wax coverage exceeding that of *S. lycopersicum* by up to seven fold. Wax composition varied in the occurrence of wax esters and triterpenoid isomers. Using a *S. habrochaites* introgression line population, we mapped triterpenoid differences to a genomic region that includes two *S. lycopersicum* triterpene synthases. Based on known metabolic pathways for acyl wax compounds, hypotheses are discussed to explain the appearance of wax esters with atypical chain lengths. These results establish a model system for understanding the ecological and evolutionary functional genomics of plant cuticles.

Most studies of tomato fruit pericarp biology have involved homogenization of the whole fruit pericarp, with its many constituent cell types, in order to isolate and study transcripts, proteins or metabolites. We coupled pyrosequencing technology with laser capture microdissection (LCM) to characterize the transcriptomes of the five principal tissues of the pericarp from tomato fruits. A total of 20,976 high quality expressed unigenes were identified, of which more than half were ubiquitous in their expression, while others were cell type specific, or showed distinct expression patterns in distinct tissues. The data provide new insights into the spatial distribution of many classes of regulatory and structural genes, including those involved in energy metabolism, source-sink relationships, secondary metabolite production, cell wall biology and cuticle biogenesis. Finally, patterns of similar gene expression between tissues led to the characterization of a previously uninvestigated cuticle on the inner surface of the pericarp, demonstrating the utility of this approach as a platform for biological discovery. The results of the transcriptomic analysis has been made publically available at the Tomato Functional Genomics Database (http://ted.bti.cornell.edu/)

We performed a proteomics study of isolated cuticles of IMG fruit harboring the *cwp*^{hir} allele. We hypothesized that we could observe the CWP protein in such a preparation if it was transported out of the epidermal cells. Our results identified 47 proteins in extracts of what appeared to be highly cleaned cuticle material, as indicated by SEM. However, the CWP protein was not observed in the cleaned cuticle. In contrast, proteomic analysis of the IMG fruit peel which included the cell layers below the epidermis did show CWP protein present.

These results indicate that the CWP protein is not localized in the extracellular cutin fraction but rather in either the epidermal or sub-epidermal cells. All of the 47 proteins identified showed expression in the outer epidermis transcriptome performed by laser dissection, described above. One of the proteins identified is the highly epidermal-specific CD1 protein which was cloned as part of the project. This is the first report of proteins isolated from cleaned cuticles and extends the proteomic results of tomato cuticles extracted by the dip method of whole fruit previously performed. We hypothesize that the proteins are synthesized in the epidermal cells and transported into the extracellular cuticular region, perhaps in association with the cuticle-embedded polysaccharide layer. The results of this study are being prepared for publication.

We observed that *Cwp*^{hab} expression causes cuticle fissuring in only *S. lycopersicum* and *S. cheesmaniae*, but not in *S. pimpinellifolium* or any of the green fruited species. This led us to undertake a QTL analysis on F_3 families of the cross of *S. lycopersicum* and *S. pimpinellifolium* lines which are homozygous *cwp*^{hab}*cwp*^{hab} and segregate for fissuring. Unfortunately, the QTL study was inconclusive and we therefore established near-isogenic tomato lines based on interspecific crosses of the wild *S. pimpinellifolium*, which is not susceptible to microfissuring in the presence of *CWP* gene expression, with both the cultivated tomato and the wild species *S. cheesmanii*, both of which are susceptible to microfissuring. Following successive generations of selection we have finally succeeded in establishing stable F_5 near-isogenic lines derived from a three-species cross that all harbor the *cwp*^{hab}*cwp*^{hab} introgression from *S. habrochaites* and that differ in their susceptibility to microfissuring. This material is being studied for differential gene expression and QTL mapping by genotyping by sequencing and the results are not yet available at the time of writing this report.

Reviewed publications

Matas, A.J., Yeats, T.H., Buda, G.J., Zheng, Y., Chatterjee, S., Tohge, T., Ponnala, L., Fernie, A.R., Adato, A., Aharoni, A., Stark, R., Fei, Z., Giovannoni, J.J. and Rose, J.K.C. (2011) Tissue and cell type specific transcriptome profiling of expanding tomato fruit provides insights into metabolic and regulatory specialization and cuticle formation. The Plant Cell 23: 3893-3910.

Yeats, T.H., Buda, G.J., Wang, Z., Chehanovsky, N., Moyle, L.C., Jetter, R., Schaffer, A.A. and Rose, J.K.C. (2012) The fruit cuticles of wild tomato species exhibit architectural and chemical diversity, providing a new model for studying the evolution of cuticle function. The Plant Journal 69: 655-666.

Yeats, T.H., Martin, L.B.B., Viart, H. M-F., Isaacson, T., He, Y., Zhao, L., Matas, A.J., Buda, G., Domozych, D.S., Clausen, M.H. and Rose, J.K.C. (2012) The identification of cutin synthase: formation of the plant polyester cutin. Nature Chemical Biology 8: 609-611.

Yeats, T.H., Huang, W., Chatterjee, S., Viart, H. M.-F., Clausen, M.H., Stark, R.E. and Rose, J.K.C. (2013) Biochemical characterization of an ancient family of cutin synthase (CUS) proteins that are conserved among land plants (The Journal of Biological Chemistry, under review).

Book chapters and reviews

Ruiz-May, E. and Rose, J.K.C. (2013) Cell wall architecture and metabolism in ripening fruit and the complex relationship with softening. In 'The Molecular Biology and Biochemistry of Fruit Ripening'. Eds. J.J. Giovannoni, M. Poole, G. B. Seymour and G.A. Tucker: Pub. Wiley (In press).

Yeats, T.H. and Rose, J.K.C. (2013) The formation and function of plant cuticles. (Submitted, Plant Physiology).

Papers in preparation

Chehanovsky, N. et al, Schaffer, A.A. Effect of temperature stress on fruit microfissuring, and expression and alternative splicing of the *CWP* gene in tomato.

Chehanovsky, N., Frankel, R., et al, Schaffer, A.A. The molecular evolutionary event leading to gene silencing of *CWP* in domesticated tomato.

Frankel, R., et al, Schaffer, A.A. Proteome of tomato fruit cuticles and localization of the CWP protein

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Appendix

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The *cwp* allele of the grren fruited wild species of the tomato clade is expressed whereas the alleles of the colored fruited wild species are not, allowing for non-fissured cultivated tomato fruit that do not readily dehydrate. By performing an interspecif promoter sequencing and analysis we identified two deletions in the *cwp* promoters of the three colored fruited species and showed that these deletions are responsible for the gene silencing. The identification of the transcription factor responsible, via IY hybrid strategy is in progress.

3. Frankel, R., et al, Schaffer, A.A. Proteome of tomato fruit cuticles and localization of the CWP protein.

   In oreder to identify the location of the CWP protein and the region of gene transcription we performed differential proteome analysis, along with in-situ hybridization. Both strategies indicate that *cwp* is expressed in the subepidermal cells and that the protein remains there and is not exported to the extracellular cuticle. This suggests a role of CWP in pericarp cellular development rather than as a component of cuticular biomechanical structure.